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The *EDA* gene is a target of, but does not regulate *Wnt* signaling

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Abstract

Lesions in the anhidrotic ectodermal dysplasia (*EDA*) gene cause the recessive human genetic disorder X-linked anhidrotic ectodermal dysplasia, which is characterized by the poor development of ectoderm-derived structures. Ectodysplasin-A, the protein encoded by the *EDA* gene, is a member of the tumor necrosis factor ligand superfamily that forms a collagen triple helix, suggesting functions in signal transduction and cell adhesion. In an effort to elucidate the function of EDA in pathways regulating ectodermal development, we have analyzed promoter elements of the gene. We show here that a binding site for the lymphocyte enhancer factor 1 (Lef-1) transcription factor is active. In electrophoretic mobility shift assays, Lef-1 specifically bound to its site in the *EDA* promoter. Over-expression of both Lef-1 and β-catenin significantly increased *EDA* transcription in co-transfection studies. In addition, indirect stabilization of endogenous β-catenin stimulated *EDA* transcription 4- to 13-fold. This is the first direct evidence of a relationship between EDA and the *Wnt* pathway. We have also investigated whether EDA might function in a feedback loop to modulate *Wnt* signaling. Over-expression of EDA neither stimulated basal transcription of *Wnt*-dependent genes, nor inhibited *Wnt*-dependent activation of transcription. Taken together, our results demonstrate that *Wnt* signaling does control *EDA* gene expression, but ectodysplasin-A does not feedback on the *Wnt* pathway.

Keywords: Ectodysplasin; Anhidrotic ectodermal dysplasia transcription; Ectodermal development; Lymphocyte enhancer factor 1/β-catenin

1. Introduction

Anhidrotic ectodermal dysplasia (EDA; Online Mendelian Inheritance in Man, 2000: 305100) is a recessive X-linked genetic disorder caused by lesions in the *EDA* gene and characterized morphologically by rudimentary teeth, sparse hair, and absent eccrine sweat glands. We have recently cloned the *EDA* gene (Kere et al., 1996) and its mouse counterpart (Srivastava et al., 1997), *Tabby*, deficiency in which causes similar abnormal development of teeth, hair follicles, and sweat glands. The range of the defects suggests that the gene has a pivotal function in the primary initiation stage of skin appendages.

Indirect evidence suggests that EDA might interact with the *Wnt* pathway during hair follicle initiation. *Wnt* signaling directs cell-fate choices during development in many organisms (Arias et al., 1999), and its downstream effectors, Lef-1, a member of the Lef/Tcf family of transcription factors, and β-catenin (Eastman and Grosschedl, 1999), have both been implicated in the regulation of epidermal development. Gat et al. (1998) observed that overexpression of β-catenin induces de novo morphogenesis of hair follicles, whereas Lef-1 null mice (van Genderen et al., 1994) have fewer follicles.

One approach to test more directly for connections with the *Wnt* pathway starts by examining the *EDA* gene promoter. Our initial studies (Pengue et al., 1999) revealed three regions of the promoter responsible for successively more specific levels of control. In particular, 5′ of two Sp1 sites that drive basal transcription is a consensus binding site for Lef-1. Here we report that the site is active, supporting EDA as a target of the *Wnt* pathway. We have also investigated whether EDA might in turn modulate the action of Lef-1 and β-catenin, but find no evidence for such reciprocal feedback signaling.

2. Materials and methods

2.1. Plasmids and constructs

p1625 (Pengue et al., 1999) is a promoter reporter

Abbreviations: EDA, anhidrotic ectodermal dysplasia; TNF, tumor necrosis factor; Lef-1, lymphocyte enhancer factor 1; TCF, T-cell factor; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; GAPDH, glyceraldehyde phosphate dehydrogenase; cpm, counts per minute; GSK3-beta, glycogen synthase kinase 3-beta; APC, adenomatous poliposis coli; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; BMP, bone morphogenetic protein; SHH, sonic hedgehog

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construct containing 1.6 kb of genomic sequences upstream of the transcription start site and part of the EDA cDNA sequence fused to the luciferase gene. The reporter vectors, pTOPFLASH and pFOPFLASH, which contain multimerized wild type (pTOPFLASH) or mutated (pFOPFLASH) TCF/Lef-1 binding sites and the thymidine kinase promoter fused to the luciferase gene, were provided by Dr R. Fodde (Korinek et al., 1997). Lef-1 (pLef-HA) and β-catenin (pBATβ) expression plasmids were obtained from Dr R. Grosschedl (Giese and Grosschedl, 1993); EDA-A1 expression plasmid (pCMV5-EDA-A1), from Dr J. Kere (Ezer et al., 1999); Wnt-1 ligand construct, pMV7-Wnt-1, from Dr D. Sussman (Song et al., 2000).

The Quickchange mutagenesis kit (Stratagene) was used to create *EDA* promoter reporter constructs with the following mutations in the Lef-1 binding site (5′-CTTTGAA-3′; mutated bases are underlined): 5′-CGGAGAA-3′ (plefmu12); 5′-ATTTCGAA-3′ (plefmu34); 5′-AATTCAA-3′ (plefmu56). All constructs were sequence verified.

2.2. Cell culture and DNA transfection

HeLa, 293, HaCaT, MCF-7, and PAM212 (Yuspa et al., 1980) cell lines were maintained in Dulbecco's Modified Eagle's Medium (LifeTechnologies) supplemented with 10% fetal calf serum and 2 mM L-glutamine. The human colon adenocarcinoma cell line SW480 (American Type Culture Collection, Manassas, VA) was maintained in Leibovitz's medium (LifeTechnologies) supplemented with 10% FCS and 2 mM L-glutamine. All cell lines used are epithelial-derived and were shown by reverse transcriptase polymerase chain reaction (RT-PCR) to express EDA at levels similar to other cells (Kere et al., 1996; Bayes et al., 1998).

For studies of *EDA* promoter function in HeLa, HaCaT, and 293 cell lines, transfections were carried out using the manufacturer's protocol for Superfect transfection reagent (Qiagen). In MCF-7, SW480, and PAM212 cell lines, the DOTAP liposomal transfection reagent (Roche) was used according to the manufacturer's recommendations. Titration experiments determined optimum amounts of each reporter construct and expression plasmid for cotransfection. A plasmid expressing β-galactosidase was cotransfected and empty vectors were used to adjust the amount of DNA in all cases. After 24–48 h, all cell lines were harvested with Reporter Lysis Buffer and luciferase activity was measured using the Luciferase Assay System (Promega). For the β-catenin stabilization experiments in 293 and HeLa cells, transfected cells were treated with 1 M lithium chloride to a final concentration of 25 mM 24 h prior to harvesting. All transfection data were normalized to β-galactosidase activity and protein concentration.

Expression of the EDA protein (ectodysplasin) in transfected cells was confirmed by Western blotting. A total of 24–48 h after transfection cell extracts were prepared in RIPA buffer and separated by SDS-polyacrylamide gel electrophoresis. Antibody to a C-terminal polypeptide (SKHTTFFGAIRLGEAPAS) and an anti-D-EDA antibody (Ezer et al., 1999) were used as the primary antibodies in Western blotting.

2.3. Real time quantitative RT-PCR experiments

Total RNA was isolated from transfected PAM212 mouse keratinocyte cells using Trizol reagent (Roche). Reactions were set up in triplicate with the TaqMan One Step RT-PCR Master Mix reagents kit and cycled according to Applied Biosystems, Inc. Forward primer: 5′-ATTGTCTTATGCTAGGTTCATGAGACTT-3′; reverse primer: 5′-CCCATCTCCTCAAAAAGAAATACATT-3′; and fluorescently labeled TaqMan probe (5′-[FAM]TTCCAATTGCCTAGCTTCAGGGTACCAGG-3′) were designed using the Primer Express software (Applied Biosystems, Inc.). PCR products were detected with an ABI7700 System.

To quantitate the amount of product, a standard curve was generated for each primer set using known amounts of RNA from untransfected cells, and the threshold cycle (C_T) value for each reaction was used to calculate the amount of specific product. For each transfected sample, the amount of EDA product was divided by the amount of GAPDH product and the values compared to determine the fold-induction of EDA.

2.4. Electrophoretic mobility shift assays

Gel shift assays were performed with double-stranded oligonucleotides from the EDA promoter region covering nt −382 to −353. The GSlef oligonucleotide contains the wild type Lef-1 binding site. GSmlef34 and GSmlef12 contain a mutated Lef-1 binding site identical to the ones in the plefmu34 and plefmu12 promoter reporter constructs (see above). Nuclear extract was prepared from HeLa cells, and DNA binding assays were performed as described (Majello et al., 1990) with 2 μg of extract and 10,000 cpm of labeled oligonucleotide per reaction.

In competition experiments, unlabelled competitor oligonucleotide (10–1000-fold molar excess) was included in the preincubation reaction with nuclear extract. For antibody 'supershift' experiments, rabbit antiserum specific for mouse Lef-1 (1, 2, or 4 μl, from R. Grosschedl; Travis et al., 1991) or β-catenin (0.5 or 1.0 μg; Santa Cruz Biotechnology, Inc.) was incubated with nuclear extract for 30 min on ice before addition of the radiolabelled oligonucleotide.

3. Results

3.1. Lef-1 augments EDA promoter function in transfection studies

Because the Lef-1 transcription factor has been implicated in the development of ectodermal structures (van Genderen et al., 1994; Kratchowil et al., 1996), the function-

ality of the Lef-1 binding site in the *EDA* promoter was tested. Lef-1 and β-catenin form a functional transcription factor complex as a result of *Wnt* signaling, and their overexpression can bypass a *Wnt* signal (Behrens et al., 1996). Thus, we cotransfected HeLa and HaCaT cells with wild-type *EDA* promoter construct p1625 and Lef-1 or β-catenin plasmids either alone or in combination (see Section 2.2).

The p1625 construct itself supports easily measurable levels of transcription (Pengue et al., 1999). When both Lef-1 and β-catenin were transfected with p1625, transcription from the *EDA* promoter was further stimulated to very high levels (8—9-fold in HeLa cells and 7-fold in HaCaT cells; Fig. 1). Considerable activation (4.5—5-fold) was observed in both cell lines when Lef-1 alone was cotransfected with p1625. This could be explained by a likely association of over-expressed Lef-1 protein with endogenous β-catenin. In any case, the β-catenin expression plasmid alone did not significantly induce transcription from the *EDA* promoter, so that the observed stimulation of *EDA* transcription by both expression plasmids together was more than the combined effects of each plasmid.

To assess the specificity of the binding, promoter constructs containing mutations in the Lef-1 binding site were also constructed and tested in transfection studies in HeLa cells. The mutant constructs were unresponsive to Lef-1 and β-catenin overexpression (Fig. 2), and exhibited about 50% of the wild type transcriptional activity of the *EDA* promoter in the presence of endogenous Lef-1 (data not shown).

3.2. Overexpression of Lef-1 increases endogenous levels of the EDA transcript

Ectodysplasin is normally expressed at very low levels and is difficult to detect. For this reason, we used a mouse keratinocyte cell line, PAM212, in which we are able to detect the *EDA* transcript by RT-PCR, to determine the effect of Lef-1 and β-catenin overexpression on endogenous levels of *EDA*.

We performed real time quantitative RT-PCR experiments using RNA from PAM212 cells that had been transfected with Lef-1 and β-catenin expression plasmids alone or in combination. As in the other cotransfection experiments (Fig. 1), overexpression of Lef-1 or β-catenin alone increased levels of the *EDA* transcript somewhat (Table 1). However, when both Lef-1 and β-catenin are overexpressed, levels of the *EDA* transcript are increased 7-fold (Table 1), comparable to the induction of the *EDA* promoter construct seen in the transfected HaCaT cells (Fig. 1). Thus, even low levels of endogenous *EDA* expression respond significantly to Lef-1 action.

3.3. Inhibition of GSK3-β activity activates EDA transcription

GSK3-β is a critical part of a complex of proteins that negatively regulate *Wnt* signaling by targeting excess β-catenin for degradation. Lithium-ion inhibition of GSK3-β activity results in the stabilization of cytoplasmic β-catenin and the activation of Lef-1/β-catenin-responsive genes (Stambolic et al., 1996). As shown in Fig. 3, when cells were transfected with the p1625 construct, subsequent treatment with lithium chloride resulted in a 13-fold induction of transcription from the *EDA* promoter in human embryonic kidney 293 cells and a 4-fold induction in HeLa cells. Transcription from the promoter in the pTOPFLASH construct (which is responsive to Lef-1/beta-catenin; see below) was

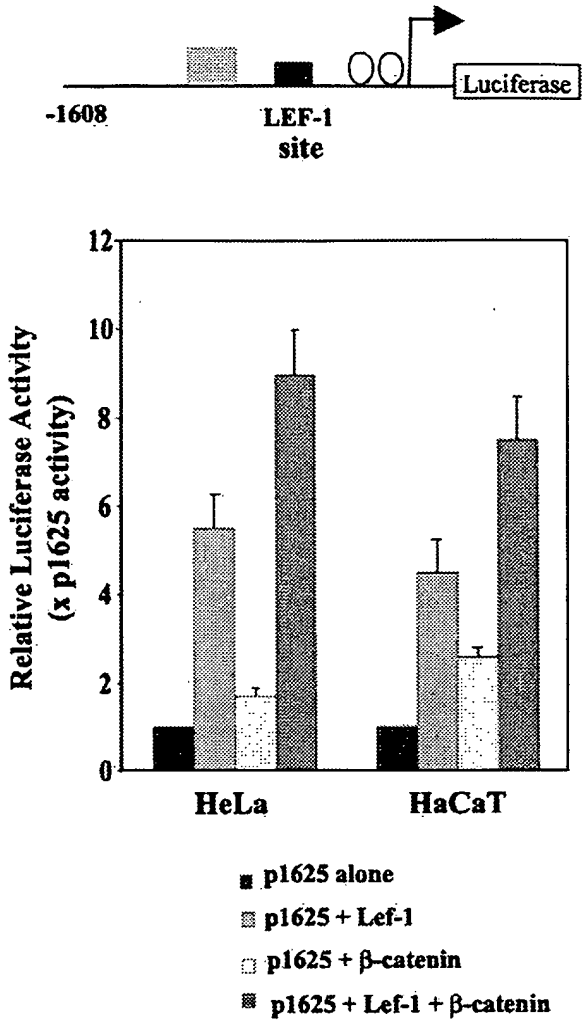

Fig. 1. Effect of Lef-1 and β-catenin on transcriptional activity of the *EDA* promoter. *Above*, the wild-type *EDA* promoter construct p1625. The transcription start site of the *EDA* gene is indicated by an arrow. Open circles and a gray rectangle represent Sp1 boxes and an *EDA* enhancer region. The Lef-1 binding site (nt −372 to −366) is indicated by a dark rectangle. *For the bar graphs*, cells were transfected (see Section 2.2) with 500 ng of p1625 alone or with Lef-1 and/or β-catenin expression plasmids. Luciferase activity was measured 48 h later. Activities were normalized to β-galactosidase activity and protein concentration and expressed relative to the activity of p1625 alone. Values are the average of three separate experiments.

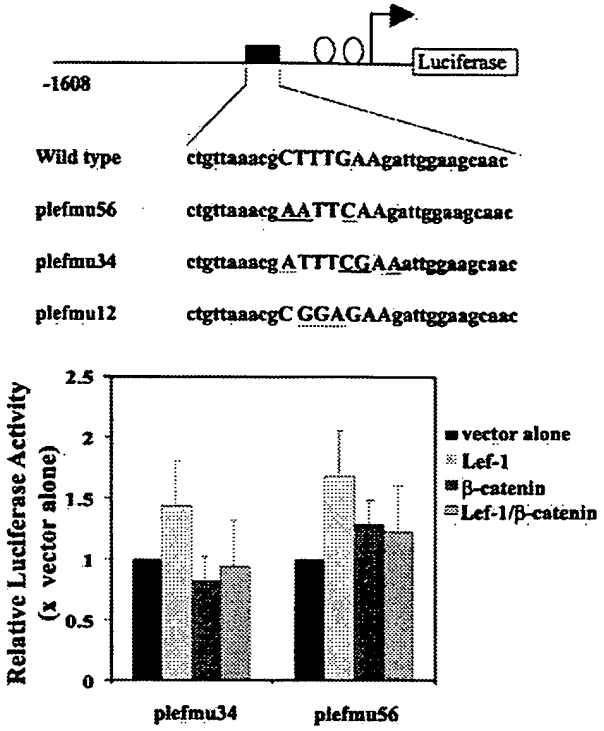

Fig. 2. Transcriptional activity of *EDA* promoter constructs containing a mutated Lef-1 binding site. *Above*, *EDA* promoter constructs, as in Fig. 1. Lef-1 core consensus binding sequence is capitalized. Bases mutated in each construct are underlined. *Below*, HeLa cells transfected with mutated promoter reporter constructs alone or with Lef-1 and/or β-catenin expression plasmids were assayed for luciferase activity and normalized as in Fig. 1. Activities are the average of three experiments and expressed relative to the activity of each reporter construct alone.

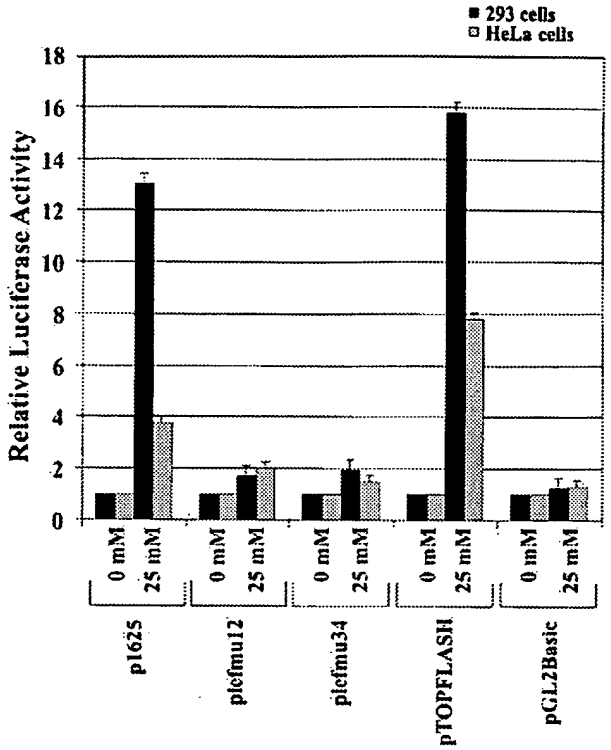

Fig. 3. Transcriptional activity of constructs in 293 and HeLa cells treated with LiCl. 293 cells and HeLa cells were transfected (see Section 2.2) with reporter constructs. LiCl (1 M) was added to a final concentration of 25 mM and luciferase activity assayed 24 h later. Activities were normalized and reported as the average of three separate experiments, as in Fig. 1. *Dark bars*, 293 cells; *gray*, HeLa cells.

stimulated almost 16- (293 cells) and 8-fold (HeLa cells). In controls, constructs containing a mutated Lef-1 binding site and the insert-less pGL2Basic vector, containing no promoter sequences, showed no induction of activity by lithium chloride treatment in either cell line (Fig. 3).

Table 1
Quantitation of EDA transcript levels by real time RT-PCR[a]

Plasmid[b]	ng EDA transcript per ng GAPDH transcript[c]	Fold induction[d]
Vectors	3.32	–
Lef-1	6.34	1.91 ± 1.27
β-catenin	10.94	3.29 ± 1.10
Lef-1/β-catenin	24.20	7.29 ± 1.76

[a] Results reported here are a representative experiment.
[b] PAM212 murine keratinocytes were transfected as described with the indicated expression vectors.
[c] Reported values were calculated using the standard curve method and are the average of three samples, normalized to the average amount of GAPDH transcript.
[d] Induction is reported relative to the amount of EDA transcript in untransfected cells.

3.4. Electrophoretic mobility shift assays of promoter DNA fragments with Lef-1

To verify that endogenous Lef-1 truly bound to the binding site in the *EDA* promoter, we performed electrophoretic mobility shift assays with HeLa cells. One shifted band was consistently observed when a double-stranded oligonucleotide containing the *EDA* Lef-1 binding site was incubated with HeLa nuclear extract (Fig. 4A, *lane 1*). The major complex was competed away by addition of increasing amounts of unlabelled oligonucleotide (Fig. 4A, *lanes 2–5*). This indicates that the major complex specifically includes Lef-1. A variable, faint second band may contain multimers of Lef-1/Tcf proteins or unstable Lef-1/Tcf-associated β-catenin.

To establish that Lef-1 specifically bound to the *EDA* promoter, and to test the notion that the larger complex might contain β-catenin, antibodies specific for Lef-1 (Travis et al., 1991) or β-catenin were added to the binding reaction. Increasing amounts of Lef-1 antibody resulted in a 'supershift' (shift to lower mobility) of both complexes (Fig. 4B, *lanes 3–5*), demonstrating that Lef-1 specifically binds to the *EDA* promoter. High levels of β-catenin antibody 'supershifted' only the larger complex and resulted in

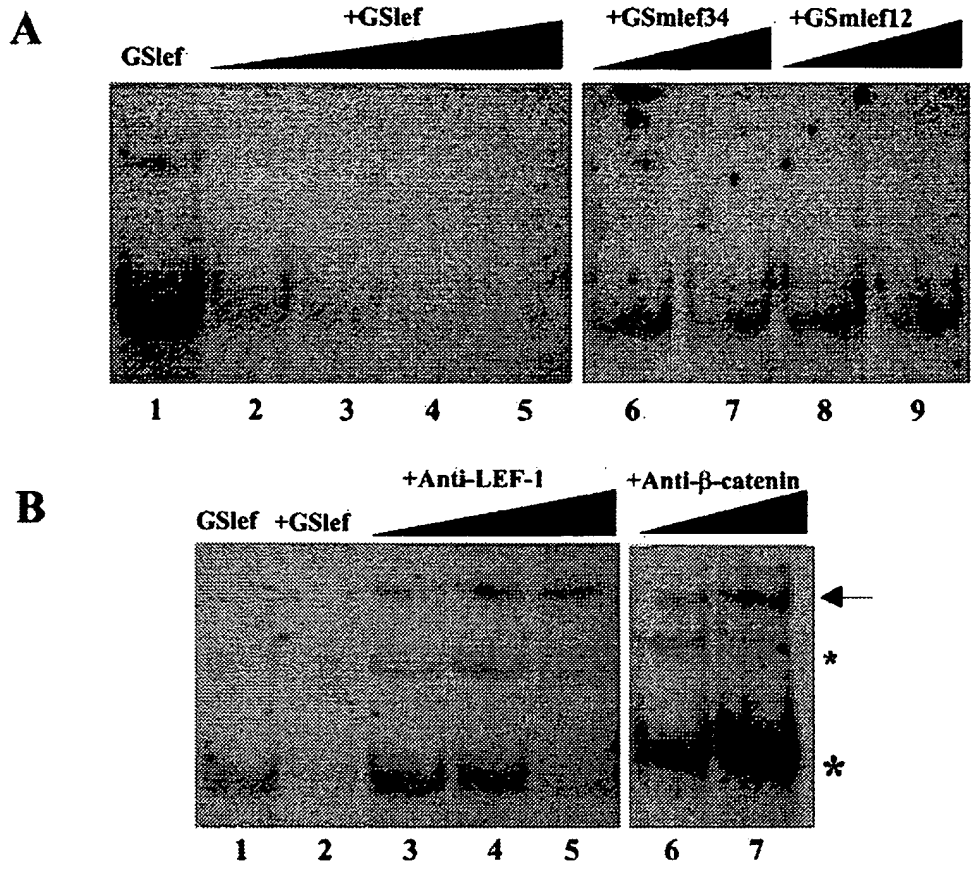

Fig. 4. Electrophoretic mobility shift assays of the Lef-1 binding site in the *EDA* promoter. Gel shift assays were performed (see Section 2.4) with double-stranded oligonucleotides containing the Lef-1 binding site from the *EDA* promoter. *Panels A* and *B, lane 1*, oligonucleotide with the wild-type Lef-1 binding site (GSlef). *Panel A, lanes 2–5*, 10-, 100-, 500- and 1000-fold molar excess of unlabelled GSlef were pre-incubated with nuclear extract before addition of labelled GSlef. *Panel A, lanes 6–9*, unlabelled GSmlef34 (*lanes 6 and 7*) and GSmlef12 (*lanes 8 and 9*), containing two different mutations in the Lef-1 binding site, were added (in 50- and 100-fold molar excess) as competitor in the pre-incubation mixture. *Panel B, lane 2*, 1000-fold molar excess of unlabelled GSlef was added as competitor. *Lanes 3–5*, 1, 2, and 4 μg of polyclonal antibody to the Lef-1 protein (Travis et al., 1991) were pre-incubated with nuclear extract before addition of labeled GSlef. *Panel B, lanes 6 and 7*, 0.5 and 1.0 μg of polyclonal antibody to the β-catenin protein were incubated with nuclear extract before labelled GSlef was added. *Panel B*, specific complexes are indicated by asterisks (*); supershifted complex, an arrow.

increased intensity of the major lower band (Fig. 4B, *lanes 6–7*). We infer that β-catenin is included in the larger, less stable complex and that addition of specific antibody titrates some of it, leaving free Lef-1 to bind to its cognate site. As further confirmation of Lef-1 binding to the *EDA* promoter, binding to the Lef-1 site was competed away by addition of increasing amounts of unlabelled oligonucleotide (10—1000-fold molar excess; Fig. 4A, *lanes 2–5*). Unlabelled oligonucleotides containing either of two different mutations in the Lef-1 binding site were not able to compete effectively (Fig. 4A, *lanes 6–9*).

3.5. Does EDA feedback to modulate Wnt signaling?

Hints of interactions of EDA with various pathways (see Section 4.2) raised the possibility that it might act in a feedback loop to modulate *Wnt* signaling. This notion can be tested using the pTOPFLASH and pFOPFLASH constructs as reporters of TCF/Lef mediated transcription (Korinek et al., 1997). The pTOPFLASH reporter construct is strongly transcribed when Wnt-1, β-catenin, and Tcf/Lef expression plasmids are cotransfected (Korinek et al., 1997; Song et al., 2000). Furthermore, in cells that are mutant in the *APC* gene (a negative effector of the *Wnt* signaling pathway), β-catenin is stabilized, and transfection experiments yield much higher transcription from the pTOPFLASH reporter gene (Morin et al., 1997). Thus, cotransfection of EDA with pTOPFLASH or pFOPFLASH in APC-containing and APC-deficient cells can provide evidence for any function of EDA in modulation of the *Wnt* signaling pathway.

3.6. Overexpression of EDA does not increase basal Wnt-dependent transcription

To test for a direct activating effect of the *EDA* gene on the *Wnt* signaling pathway, transfections included an EDA-A1 expression construct and the pTOPFLASH or pFOPFLASH construct. As shown in Fig. 5A, for MCF-7 cells,

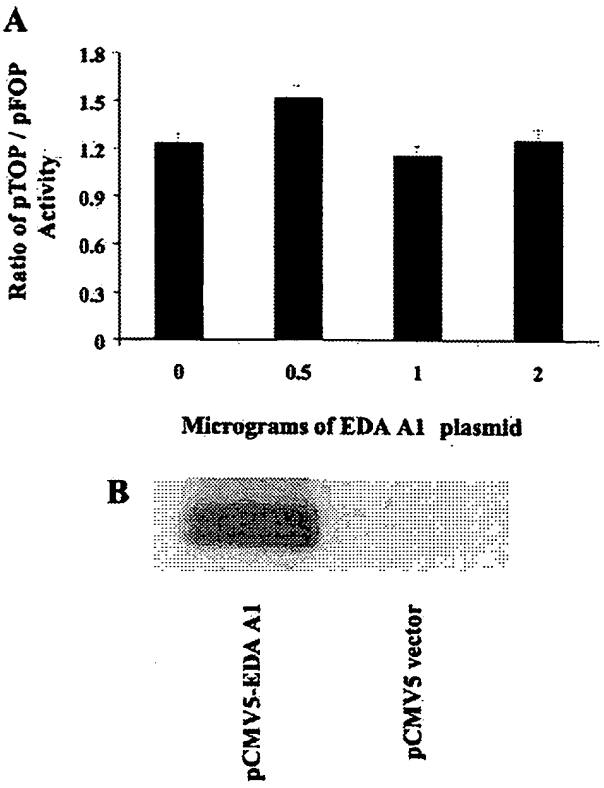

Fig. 5. Effect of EDA over-expression on basal *Wnt*-dependent transcription. *Panel A*, MCF-7 cells were cotransfected with pTOPFLASH or pFOPFLASH plasmid and pCMV5-EDA-A1 construct (see Section 2.2). Luciferase activity is the ratio of pTOPFLASH activity to pFOPFLASH activity after normalization (as in Fig. 1), averaged from triplicates. *Panel B*, western blot of transfected and untransfected MCF-7 cell extract with antibody to a C-terminal peptide of EDA (see Section 2.2).

the luciferase activities from pTOPFLASH and pFOPFLASH were very comparable (a ratio of about 1.2). This indicates that the *Wnt* signaling pathway is silent or at baseline levels in these cells. pTOPFLASH reporter gene function was not increased by overexpression of the EDA-A1 construct over a range of levels at which the EDA protein (ectodysplasin-A) was easily detected by Western blotting in extracts from the same cells (Fig. 5B). Apparently EDA has no upregulating effect on the quiescent *Wnt* pathway. The same result was seen in 293 and HaCaT cells (data not shown).

3.7. Overexpression of EDA does not decrease active Wnt-dependent transcription

SW480 cells, which carry an inactive APC protein, were used to assess possible effects of ectodysplasin on the *Wnt* pathway downstream of the level of APC action. As expected, very high expression of the pTOPFLASH reporter gene was observed in transfected SW480 cells (Fig. 6A). The introduction of the *EDA* gene at levels that produced detectable amounts of ectodysplasin-A (as in Fig. 6C) did not decrease pTOPFLASH transcription. Thus, there was no evidence for an effect downstream of APC.

To test whether EDA might have an effect upstream of the level of the *APC* gene, we carried out further transfections with transcription augmented by higher Wnt-1 levels. As expected, transfection of Wnt-1 (pMV7-Wnt-1) along with a Lef-1 expression plasmid in MCF-7 cells led to higher expression of cotransfected pTOPFLASH compared to transfection of the Lef-1 construct alone (a 3-fold increase; Fig. 6B). If EDA were to function upstream of the *Wnt* signaling pathway, an increase of pTOPFLASH transcription in the presence of higher levels of Wnt would be counteracted by higher levels of EDA; but no reduction was seen, even when EDA was highly overexpressed (Fig. 6C). Thus, there was no evidence of a role for EDA upstream of the level of APC/*Wnt* interaction.

4. Discussion

4.1. EDA as a target of Wnt signaling

Previous studies have implicated the *Wnt* pathway, and especially Tcf/Lef factors and β-catenin, in epidermal development (Gat et al., 1998; van Genderen et al., 1994; Kratchowil et al., 1996). Except for the hair keratin genes (Zhou et al., 1995), at least one of which (*HK1a*) is activated by Lef-1/β-catenin (Merrill et al., 2001), few targets of *Wnt* signaling in this system have been identified. This work presents evidence that one target gene is *EDA*.

It has been known for some time that the *EDA* promoter contains a consensus binding sequence for the Lef-1 transcription factor (Kere et al., 1996) and a mutation in this site that is associated with EDA has been described (Kobielak et al., 1998). Here we demonstrate that the site is active. The results are consistent with the general pathway in which Lef-1 forms a bipartite transcriptional activation complex with β-catenin (Behrens et al., 1996; Willert and Nusse, 1998). Electrophoretic mobility shift assays and cotransfection studies demonstrate that Lef-1 specifically binds the *EDA* promoter, and that Lef-1 and β-catenin are necessary for full activation of *EDA* gene expression. Also, inhibition of GSK3-β, which stabilizes excess β-catenin, stimulates transcription from the *EDA* promoter. Our results are consistent with the observation that expression levels of the *Tabby* transcript are decreased in the affected tissues of Lef-1 knockout mice (Laurikkala et al., 2001); and with EDA-like features in Lef-1 knockout mice (van Genderen et al., 1994).

All Tcf/Lef transcription factors recognize the same consensus sequence and at least one other factor, Tcf3, is expressed in structures affected in EDA, such as the hair follicle (DasGupta and Fuchs, 1999). Lef-1 and Tcf3 are expressed in different subsets of hair follicle cells, overlapping with *EDA* expression in both. This raises the possibility, which cannot be excluded here, that Tcf3 may also

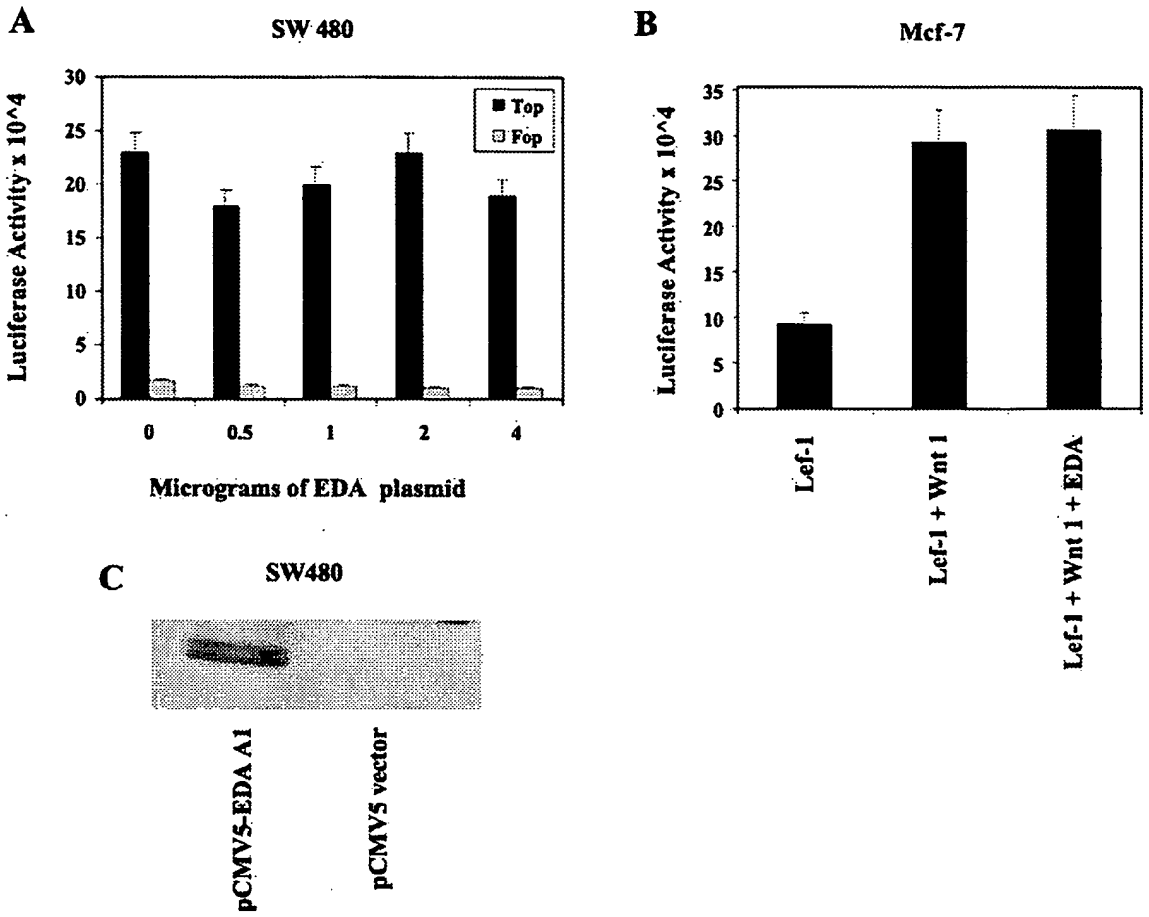

Fig. 6. Effect of EDA over-expression on active *Wnt*-dependent transcription. *Panel A*, dose-dependent transfection experiments (see Section 2.2) in SW480 cells with pTOPFLASH or pFOPFLASH and amounts of the pCMV5-EDA-A1 construct. Activities were normalized and averaged from three experiments. *Panel B*, pTOPFLASH plasmid was cotransfected into the MCF-7 cell line with the indicated combinations of Lef-1 (200 ng), Wnt-1 (1 μg), and EDA (1 μg). Luciferase activities were normalized and averaged from three experiments. *Panel C*, western blot of transfected and untransfected SW480 cell extracts with antibody against a C-terminal peptide of EDA (see Section 2.2).

regulate *EDA* gene expression in the hair follicle. Since Tcf3 acts as a repressor of gene expression in the hair follicle (Merrill et al., 2001), the potential balance of positive and negative control of *EDA* expression by Lef-1 and Tcf3 would be an intriguing explanation of the observation that the *EDA* promoter exhibits strong expression in vitro despite very low levels of expression in vivo (Pengue et al., 1999).

4.2. Interaction of EDA with signaling pathways other than Wnt

In addition to the *Wnt* pathway, sonic hedgehog (SHH; Chiang et al., 1999), bone morphogenetic proteins (BMPs; Wilson and Hemmati-Brivanlou, 1995), and EGF signaling through its receptor, EGFR (Murillas et al., 1995), are involved in epidermal differentiation. The identification of Ectodysplasin-A as a TNF ligand (Mikkola et al., 1999) implicates that signaling pathway as well in the control of epidermal appendage development and at least three potential receptors for EDA have recently been identified that are members of the TNF receptor superfamily: EDAR, recently cloned by Headon and Overbeek (1999), XEDAR (Yan et al., 2000), and TROY (Kojima et al., 2000). Consistent with its role in development, mutations in the mouse *EDAR* gene produce the downless (*dl*) phenotype, which is indistinguishable from Tabby (Monreal et al., 1999; Headon and Overbeek, 1999).

Additional hints of the involvement of other pathways come from further analyses of the *EDA* promoter. A complex region further upstream from the transcription initiation site than the Lef-1 binding site contains sequences that enhance transcription still further (Pengue et al., 1999). Suggestively, preliminary work (M. Durmowicz and C. Cui; in progress) indicates that this region includes a binding site previously noted in the *EGFR* gene (Maekawa et al., 1989), and that several protein factors that bind to the region may be responsible for tissue- and developmental stage-specific *EDA* gene expression.

Interestingly, the *BMP4* gene and the *SHH* gene are not expressed in EDAR mutant skin (Headon and Overbeek, 1999), suggesting that EDA and EDAR may be located in a pathway upstream of BMP4 and SHH. Previous studies had also shown that Lef-1 expression could be activated by BMP4 (Kratchowil et al., 1996). The sum of these observations suggested that EDA might affect the activity of Lef-1, the downstream effector of the *Wnt* pathway, either through EDAR and BMP4 or unknown pathways. Our experiments indicate that EDA does not intervene to augment or reduce *Wnt* action on the test promoter; EDA, BMP4, and Lef-1 may interact by crosstalk rather than in a single pathway.

4.3. Conclusions

EDA may be a point of convergence for a number of biochemical pathways involved in epidermal development. It is likely that EDA has dual functions, in cell-cell or cell-matrix contacts (Mikkola et al., 1999) and in signal transduction. We have shown that the regulatory networks that control the expression of the *EDA* gene include the *Wnt* pathway. Experimental results do not support a feedback loop for EDA on *Wnt* expression itself (Figs. 5 and 6), though *Wnt* also affects epidermal development in other pathways. Ongoing analysis of the enhancer region of the *EDA* promoter is identifying additional features of the way in which EDA intervenes in skin formation.

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